Chapter 5

Biological Branching Patterns

Current science research has no boundaries and knows no limits. Scientists find fractals everywhere and follow the trail of these fascinating patterns across many sciences: physics, chemistry, biology, astronomy, and earth science. In this chapter we do the same, tracing the rough edges of bacterial colonies, the scraggly outlines of termite tunnels, and the spread of root systems. Earlier chapters have shown that physical and chemical systems, formed and driven by random processes, can be very dynamic. Are living systems even more dynamic? Do patterns created by bacteria, termites, and ants result from their social nature, or are these patterns, too, the result of random processes? We try to answer these questions by applying the analysis of previous chapters.

5.1 Bacterial Colonies

In this section you will study shapes of bacterial colonies grown under various nutrient conditions. The primary questions are these: Why are the shapes of bacterial colonies grown under starvation conditions similar to patterns of non-living systems, such as electrochemical deposition and viscous fingering? Can we understand these shapes from our experience with random walks and diffusion?

The growth of bacterial colonies reflect a current research question: Does the growth pattern of the colony result from purposeful collective social behavior by the bacteria? Or can it be explained by the physics of random processes (as with a random walker model)? You will reach your own conclusions about whether the bacterial colony shapes you obtain are determined primarily by simple physics, or whether social interactions among the bacteria are necessary to create the observed patterns.

Standard laboratory practice is to grow a bacterial colony on a flat gelatinous surface with a rich source of nutrition. In this experiment, by contrast, the bacterial colonies are grown under varying nutritional conditions that approach the starvation threshold. The gel resides in a round flat dish (a standard petri dish) about 9 centimeters in diameter. To start growing the colony, you should put a small drop of bacteria in the center of the gel. Then you cover the petri dish and place it in an incubator at a standard temperature and study the pattern of the colony as it grows outward over a period of days and weeks.

Q5.1: Why would scientists, in particular those in a hospital, want grow bacteria?

What happens when the bacteria are forced to grow in starvation conditions? Can they hunt for food? What determines whether they receive nutrition? Do bacteria cooperate among themselves? What determines the shape of the colony? What shape is optimal for survival? Is the shape we see the optimal one? And what about the nutrients? Do they remain stationary in the gel waiting for the bacteria to come and graze? Or do they diffuse through the gel to areas of low concentration where bacteria have eaten the nutrients?

Q5.2: Speculate about answers to the above questions before beginning the experiment. Also, sketch the shape you expect the colony to have as it grows. Can you draw analogies between growth of bacteria in this experiment and the growth of a diffusion-limited aggregation in the **Aggregation Kit** program?

There is, of course, an innate tendency to think differently about living organisms than about "dead" objects. But now we are well aware that non-living materials can grow into beautiful, elaborate patterns. So here are some more questions:

> Q5.3: Is there an advantage to drawing an analogy between bacteria and non-living matter? List the advantages and disadvantages of "projecting" onto bacteria your own expectations about how living organisms behave. Does it make sense to ask what bacteria *want* to do? *try* to do? *plan* to do?

When you did the electrodeposition experiment, you probably had an internal mental model for what was happening. You "imagined" copper ions moving through solution. You "pictured" them attaching to the growing aggregate and being reduced (i.e., adding two electrons). You reasoned about the applied electric field and its ability to drive ions through the solution, and electrons through the external circuit. And when you compared your results to those of the **Aggregation Kit** simulation, you drew analogies between the simulation and the experiment.

In order to build a mental model to analyze the bacteria experiment, you need certain facts about bacteria and their behavior. You may also find it useful to adopt some of the code words scientists use to discuss these experiments. These words include:

- *motile*, the biology synonym for "mobile";
- *chemotaxis*, shorthand for motion of bacteria in response to chemicals in their environment;
- *viscosity*, defined in the Hele-Shaw cell experiment (HandsOn 14) as the difficulty with which one fluid moves through a narrow tube or penetrates another liquid;
- *flagellum*, a whip-like organelle ("little organ") that many bacteria use to propel themselves; plural: *flagella*.

Among the bacteria we recommend for this experiment are *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), and *Enterobacter aerogenes* (*E. aerogenes*). All are rod-like in shape with dimensions 2–3 micrometers (μm) in length and 1μm in width. They occur commonly, are usually found in the human digestive tract, soil, and unpasteurized dairy products. To grow *B. subtilis* and *E. coli* you need to incubate the agar plates at 37°C. *E. aerogenes* grows at room temperature. Typically all three strains of bacteria multiply every 20 minutes if adequate nutrition is available.

Bacteria respond differently to starvation conditions. *B. subtilis* retreats into a dormant mode by forming *endospores*, thick-coated cells that can withstand conditions that would quickly kill the normal active cell. In contrast, *E. Coli* and *E. aerogenes* die.

The wild strain of each of these bacteria (i.e., the most commonly observed strain in nature) is *motile* — it moves. Each is *flagellated*, that is, has various numbers of little whip-like arms. Under a microscope, bacteria growing on agar near the colony look like many tadpoles swimming randomly. It is not far-fetched to describe each bacterium as executing a random walk, one restricted to the neighborhood of the colony. Bacteria also excrete a fluid which can enhance their ability to move on the agar surface.

In our experiments, nutrients are supplied to bacteria by dispersing them in a gelatin-like material made from agar. Agar is a long-chain molecule made from seaweed. When dissolved in water at sufficiently high concentrations, it causes the solution to gel. In this way, agar is much like gelatin. Nutrient molecules diffuse through agar gel nearly as quickly as through water.

Bacteria respond to chemical signals. One famous example is that of slime mold. Under starvation conditions the bacteria release chemicals that result in a chemotactic response: the bacteria come together into a single colony and form a spherical growth called a *fruiting body* to survive the adverse conditions. (In biology *taxis*—pronounced "tax-iss"—refers to motion by an organism in response to a stimulus. *Chemotaxis* is motion in response to a chemical stimulus. *Phototaxis* is motion in response to light. These terms are similar to *chemotropism* and *phototropism*; however a *tropic* response can include growth as well as motion.)

While it is known that bacteria communicate chemically, it is difficult to isolate the chemicals they use, or to distinguish purposeful organization resulting from this chemical communication. As an example of the distinction: in electrochemical deposition, beautiful patterns emerge as the ions aggregate; moreover, the atoms "communicate" chemically. In spite of this, we do not say that copper atoms behave socially!

Many of the HandsOn activities throughout this chapter also require the use of the computer to analyze fractal dimensions of objects. We will only label an activity as SimuLab if that activity is strictly focussed on computer simulation or analysis.

HandsOn 17: Modeling a Rough Surface

As part of your data analysis you will measure the fractal dimension of the surface, or perimeter, of the bacterial colony. The question is whether the colony interface may reflect social interactions, or whether the spread of the colony is effectively random.

A random surface can be generated by the following random walk exercise. On a piece of graph paper, draw a horizontal line across the middle. This is your $y = 0$ line. Make a mark at the left-most point of the axis. This is your origin ($x = 0$, $y = 0$). Move your marker over one unit to $x = 1$. Flip a coin. If heads results, move up to $y = +1$; if tails, move down to $y = -1$. Move your marker over to $x = 2$. Flip the coin. If it is heads, move y up one; if tails, move y down one. Repeat this process moving your marker over one unit on the x-axis each time (the x-axis represents time), and up or down one unit in the y-direction, depending on the result of the coin flip. For example, if you flip THHTTTHTHHT then the connected points make a graph like Figure 5.1.

The result is a random surface. Connect your points with dark magic marker, scan it into the computer with a contrast setting so that the grid vanishes, and find the dimension using the **Fractal Dimension** program.

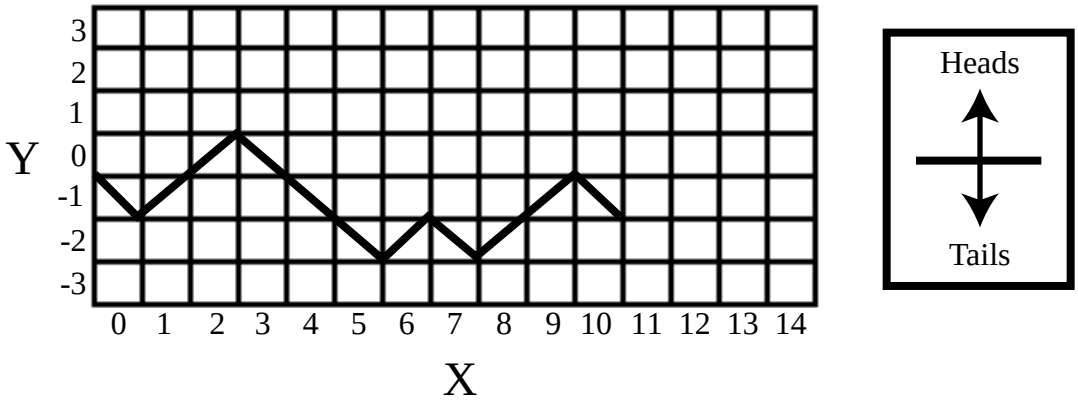

Figure 5.1: Generation of a random surface using coin flips, heads (H) or tails (T). This surface results from the sequence THHTTTHTHHT.

Q5.4: What is the fractal dimension of your rough surface?

Q5.5: What happens to the surface if we change to the following rule: flip two coins, move up only if you get two heads and move down only if you get two tails.

END ACTIVITY

A common model of molecules in gases and liquids shows particles constantly in motion, dancing around due to thermal disturbances. In a uniform gas or liquid nothing changes, on average, because of this motion; on average, equal numbers of molecules move in and out of a given volume. Since the motion is random, like that of a random walker, there is no "current," no *net* directed flow of molecules in a given direction. This is a picture of a medium that is "at equilibrium."

Now extend this mental model to the case in which a concentration difference exists. For example, suppose we have a barrier between two containers of water. The container on the left has salt dissolved in the water; the one on the right is pure distilled water. We now raise this barrier carefully, so as not to cause fluid motion.

Q5.6: What happens to the saltiness on the two sides of the *unified* chamber? Is there a net motion of salt ions from one side to the other? Why? Does the random walk of the molecules in solution contribute to this?

When a concentration difference occurs between regions of the same medium, that medium is no longer in equilibrium. The result is a net diffusion of particles from the high-concentration region to the low-concentration region.

Q5.7: As the bacterial colony grows, it eats the nutrients that are located closest to it (near its interface with the gel). This results in a lowered concentration of nutrient near the interface. But the agar gel began as a homogeneous medium with a uniform concentration of nutrients. What happens to the nutrients in the gel? Do they diffuse toward the depleted region? Will the colony still be able to continue to feed even if it does not grow?

The HandsOn experiments throughout this chapter are a little different from previous chapters. Instead of the normal boxed questions you are used to seeing, you will find that many of the questions are embedded throughout the experiments. These questions should be answered as part of a student laboratory report.

In the experiment HandsOn 20 you will be growing bacteria under various nutritional conditions. In HandsOn 18 you will prepare agar plates to grow the bacteria, and in HandsOn 19 you will create streak plates to produce individual colonies of genetically identical bacteria.

HandsOn 18: Bacterial Growth Preparation

Detailed instructions for this experiment can be found on our Web site.

To minimize contamination of your experiment by the bacteria that live on and around us, please:

1. Wash your work area with a disinfectant detergent (e.g., vesphene) before beginning your experiment.
2. Wash your hands and wear gloves for the experiment.
3. Work within 20 cm of a Bunsen burner flame.
4. At the end of your experiment, wash your work area and your hands.

This experiment requires the following supplies and equipment:

- incubator (if available)
- autoclave for sterilization
- Bunsen burner with tubing (gas supply)
- balance (preferably accurate to 1/100 g)
- petri dishes
- laboratory gas lighter (striker)
- scissors
- markers or wax pencils
- nylon gloves
- goggles
- aluminum foil
- autoclave gloves
- beakers

- graduated cylinders
- inoculating needles and loops
- weighing dishes
- glass stirring rods
- parafilm
- autoclave tape (optional)

Chemicals for Nutrient Agar:

- Bacto Agar
- Bacto Peptone
- dibasic potassium phosphate (K_2HPO_4)
- monobasic potassium phosphate (KH_2PO_4)
- Sodium Chloride (NaCl)
- distilled water (H_20)

Chemicals for an Alternative Nutrient Agar Luria-Bertani (LB):

- Bacto Agar
- Bacto Tryptone
- Sodium Chloride (NaCl)
- Yeast extract
- distilled water (H_20)
- Bacterial samples such as *Bacillus subtilis*, *Escherichia coli*, and it Enterobacter aerogenes.

Bacteria can be purchased from supply companies (e.g., Carolina Biological Supply Company or Difco Laboratories). Specific bacteria can sometimes be obtained from stock centers. B. subtilis is available from the Bacillus Genetic Stock Center at Ohio State University and E. coli is available from the stock center at Yale University. Both stock centers provide the bacteria free to educational institutions. See the "Yellow Pages" at the end of the book for information on suppliers.

Preparing Nutrient Agar Plates

Bacteria grow on nutrient agar. The formulas in this text are given for one liter solutions; smaller volumes than a liter can be made by scaling down the quantity of each ingredient by the same ratio as the volumes. For instance, if the formula for one liter of nutrient agar calls for 10.0 grams of agar, use 2.5 grams to prepare 250 milliliters of solution. To vary the nutrient levels in this experiment, vary the Bacto Peptone levels in the solution. The formula for the nutrient agar with 1.0 g/L of Bacto Peptone is:

Bacto Peptone Medium:

- 1.0 liter of distilled water
- 10.0 grams/Liter Bacto Agar
- 5.0 g NaCl
- 5.0 g K_2HPO_4
- 2.0 g KH_2PO_4
- 1.0 g Bacto Peptone

For streak plates, you can use a concentration of 1.0 g/L Bacto Peptone. There are many other kinds of nutrient agar that can be used to obtain individual colonies. Since bacteria grow differently on different kinds of nutrient agar, you may find that one works better than another for you. One standard nutrient agar is Luria-Bertani (LB).

Luria-Bertani (LB) Medium:

- 1.0 liter of distilled water
- 15.0 g Bacto Agar
- 10.0 g NaCl
- 10.0 g Bacto Tryptone
- 5.0 g Yeast Extract

For this procedure, you will be using an autoclave. An autoclave is designed to produce temperatures and pressures that will completely sterilize objects. It is important that you use gloves designed for use with the autoclave so that you do not injure yourself.

1. Weigh out the ingredients from either list, and place them in a beaker. Also place in the beaker a glass stirring rod to be sterilized. The stirring rod will be used after autoclaving. Agar will not dissolve into solution until the solution has been heated. Cover the beakers with aluminum foil (shiny side facing the inside of the beaker), and put the nutrient agar in an autoclave. The time necessary to sterilize the solution depends on your particular autoclave. It is a good idea to place autoclave tape on your beakers if it is available. Autoclave tape has stripes on it that are originally light colored but turn black when exposed to the temperature and pressure needed for sterilization. Thus, you can be assured that the sterilization process was successful if the stripes on the autoclave tape change color.

2. After sterilization is complete, remove the beakers wearing autoclave gloves. Remember that the autoclave operates at 121.6°C (250°F), and a steam pressure of 15 lbs per square inch (psi). The solution which comes out is very hot. Be careful!

   After removal from the autoclave, allow the beakers to cool enough that they can be comfortably handled while wearing vinyl gloves. If the agar is allowed to cool too much, it will begin to solidify. If the nutrient agar is poured while it is too hot, there will

be excessive condensation on the cover of the petri dish. Moisture on the cover will adversely effect results of later steps.

3. Stir the nutrient agar using the stirring rod which was autoclaved with the agar. Then pour agar into the petri dish until the agar just covers the bottom of the dish (approximately 20 ml). Place the petri dish cover on the petri dish immediately and allow the agar to solidify (fully gel). After the plate of agar has solidified, turn the plates upside down and let them sit for 48 hours at room temperature to dry. Drying eliminates excess water in the agar and reduces the amount of condensation.

END ACTIVITY

HandsOn 19: Streaking and Innoculating Plates

Detailed instructions for this experiment can be found on our Web site.

Cultures ordered from a supply company or stock center will probably not consist of genetically identical bacteria. The bacteria will all be of the same species, and available as a single strain. However, random mutations may still exist due to the large number of bacteria present. To obtain a source of genetically identical bacteria, streak plates are used. Streaking a plate allows the bacteria to be spread out so that a single bacterium can be isolated from all other bacteria. This technique is called streaking for individual colonies. Since bacteria are so small, you will not be able to see that isolated bacterium. However, that bacterium will reproduce itself by binary fission (typical division time is on the order of 20 minutes), resulting in bacteria which are genetically identical to the original bacterium and to each other. These bacteria are visible as a small round colony growing where there had been one isolated bacterium. This method allows you to use the individual colony repeatedly and expect similar results.

There are several acceptable streak plate methods. The method described here is called the "T" streak and is one of the easiest.

1. Light a Bunsen burner in your bench space. To maintain sterile conditions, inoculation should occur within 20 cm of the flame. Wait 20 seconds before opening the petri dish and inoculating. This gives the flame time to sterilize the local air. Remember

that you want to achieve sterile conditions. Do not work with the plate close to your face. This will violate the sterile environment.

2. Use a marker or wax pencil to draw a T on the bottom of a plate of nutrient agar. This divides the plate into three sections (Figure 5.2. One section covers one half the plate. The other half is divided into two quarters.

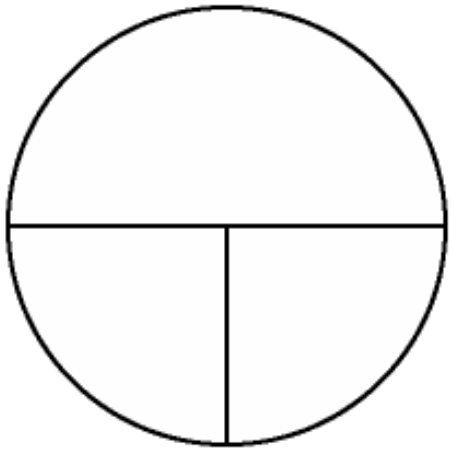

Figure 5.2: Draw a "T" on the bottom of your petri dish as shown.

3. Sterilize the inoculating loop (Figure 5.3), by holding its tip in the flame until it turns red.

4. Lift up the lid of the plate you will be inoculating and poke the inoculating loop through the agar close to the side of the petri dish to cool it. This prevents the heat from killing the bacteria sample you want to use. The heat will not harm the agar. Try to lift the lid of the plate up only as much as is necessary to put the loop inside. If you completely remove the lid, it can become contaminated with bacteria from the environment.

5. Touch the loop to the edge of the colony growing on the plate. Then take the loop and place the lid securely back on the plate.

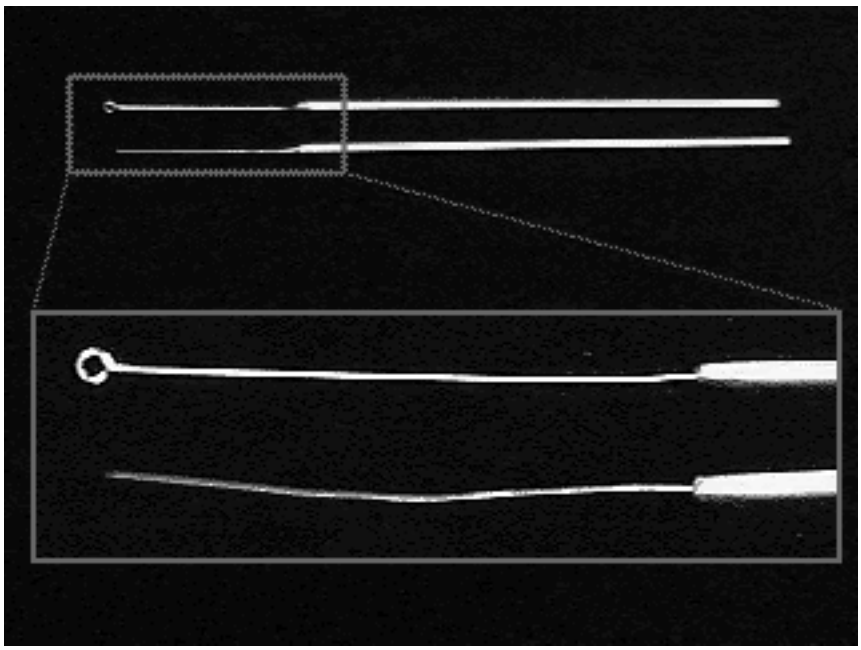

Figure 5.3: Wire innoculating loop and needle used in this experiment.

6. Set the plate you will be streaking so that its bottom is sitting on the bench top and you can see the T clearly. The largest section should be at the top. Carefully lift up the lid and touch the inoculating loop to the upper left hand corner of the largest section of the plate. Move the loop from left to right, back and forth, across the surface of the agar. See Figure 5.4. Since nutrient agar is a gel with properties similar to jello, do not push down with the loop or you will gouge the agar.

7. Replace the lid of the petri dish and flame the loop again to kill any remaining bacteria on it. Rotate the plate 90 degrees counterclockwise. Carefully lift the lid slightly and touch the loop into the left side of the plate which contains the area you streaked in the previous step. Move the loop across the surface of the agar until it is in the smaller section in the upper right of the plate. Within that quarter of the plate, move the loop back and forth across the agar surface (Figure 5.5).

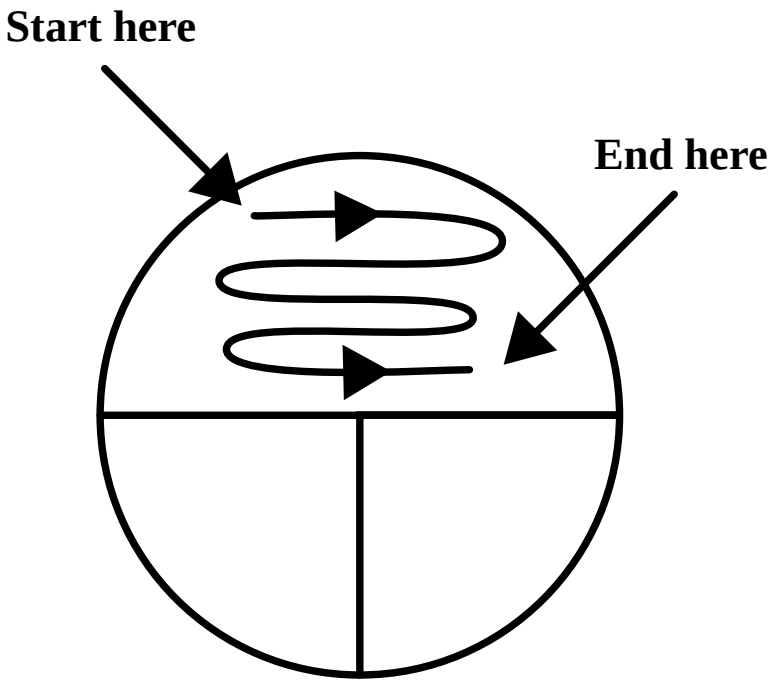

Figure 5.4: Touch the inoculating loop to the upper left hand corner and then move it across the agar from left to right as shown.

8. Repeat STEP 7 as shown in Figure 5.6.

9. Replace the lid of the petri dish and flame the loop again to kill any remaining bacteria on it.

10. Seal the petri dish with a layer of parafilm around the edge. This keeps the agar from drying out while it is in the incubator. Incubate the streak plate at 37°C until you can see individual colonies. Make sure to keep an open beaker of water in the incubator. Periodically check that the beaker has water in it—do not let it run dry. The water will maintain a constant level of humidity (100%) in the incubator. See Figure 5.7.

Once you have a streak plate with individual colonies, you should inoculate, from an individual colony, nutrient agar plates containing various concentrations of nutrients.

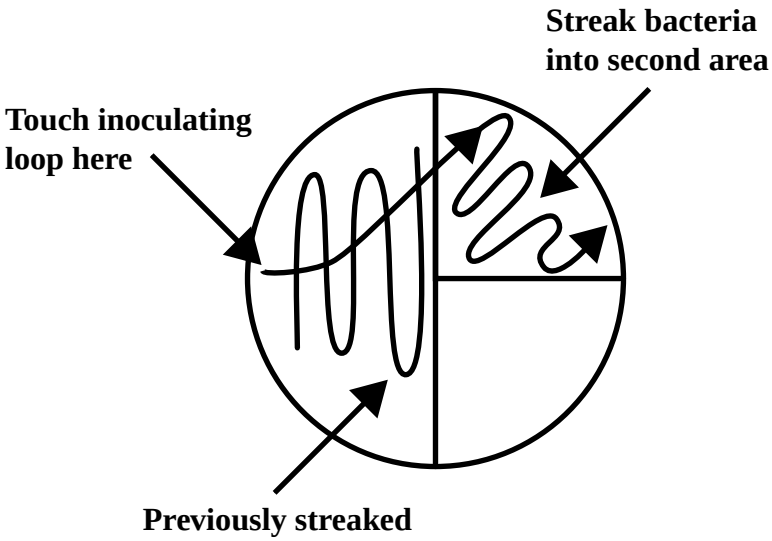

Figure 5.5: Touch the loop to the area previously streaked and then move the loop across the agar as shown.

1. As in plate streaking, light a Bunsen burner in your bench space and work within 20 cm of the flame to maintain sterile conditions. Again, wait 20 seconds before opening the petri dish and inoculating.

2. Use a marker or wax pencil to place a dot in the center of the outside bottom of the petri dish of nutrient agar. Now turn the plate over so that the bottom is sitting on the bench top. Notice that you can see the dot you just made through the agar. This will help you place the bacteria on the surface of the nutrient agar's center.

3. Sterilize the inoculating needle by placing the tip of the needle in the flame. Keep the tip there until the metal turns red.

4. Choose a streak plate containing an individual colony. Lift up the lid of the streak plate you will be inoculating from, and poke the inoculating needle into the agar close to the side of the plate to cool it. This prevents the heat from killing the bacteria sample

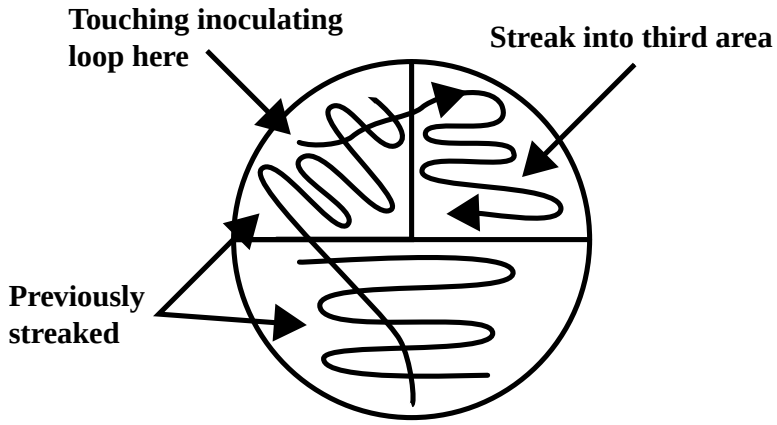

Figure 5.6: Touch the loop on the previously streaked area. Then move the loop across the agar onto the third area as shown.

you want to use. The heat will not harm the agar. Lift the lid of the plate up only as much as is necessary to put the needle inside. If you completely remove the lid, it may become contaminated with bacteria from the environment.

5. Touch the inoculating needle to an individual colony growing on the plate. Take care not to stab the inoculating needle down into the agar. Then remove the inoculating needle from the plate and place the lid securely back on the plate.

6. Carefully lift up the lid of the plate you are inoculating onto. Touch the inoculating needle to the very center of the surface of the nutrient agar. The dot you drew on the bottom should make it easier to locate. Be careful that you do not stab the inoculating needle into the nutrient agar.

7. Place the lid on the plate, and flame the inoculating needle to kill any remaining bacteria.

8. Seal the plate with a layer of parafilm around the edges. This keeps the nutrient agar from drying out while it is in the incubator. Make sure to keep an open beaker of water in the incubator. Periodically check that the beaker has water in it – do not let

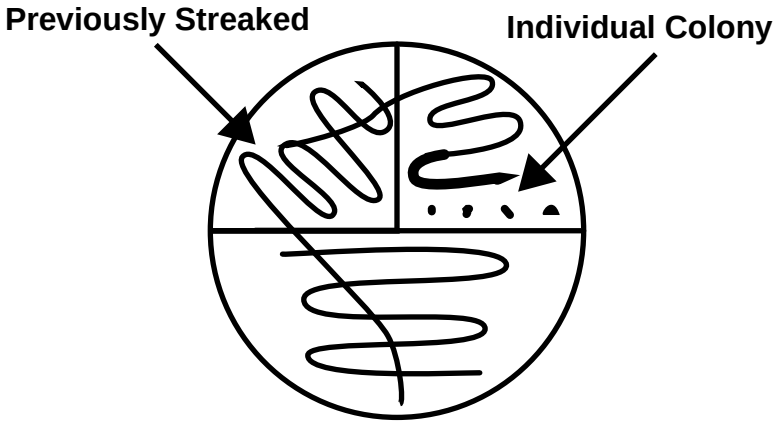

Figure 5.7: Incubate the streak plate until you can see individual colonies. Make sure to keep an open beaker of water in the incubator.

it run dry. The water will maintain a constant level of humidity (100%) in the incubator.

Caution: When disposing of unwanted bacterial colony plates, you must first autoclave them. This will kill the bacteria, and make the plates safe for disposal in a regular trash bag.

END ACTIVITY

HandsOn 20: Growth of Bacteria Under Starvation Conditions

As mentioned above, *B. Subtilis*, *E. Coli*, and *E. Aerogenes* are all commonly-occurring bacteria. In fact, we are surrounded by so many different bacteria in our environment that to grow a single strain requires careful attention to avoid *contamination* (infecting the bacteria you are growing with another strain). Always take standard laboratory precautions and wear gloves when handling the bacteria and agar plates.

1. Follow the instructions in HandsOn 18 to make your agar plates.
2. Follow the instructions in HandsOn 19 to inoculate and incubate your agar plates.
3. In addition to the single point inoculation experiments described in HandsOn 19, do an experiment in which two colonies compete on a single plate. On each of two plates, inoculate at two points, separated by 0.5 centimeter on one plate and by 1 centimeter on the other plate.
4. Measure the diameter of each bacterial colony daily and record it on a chart in your lab book.

Data Analysis

1. Record a digital image of the colony with a digital camera or scanner. It may look something like Figure 5.8.

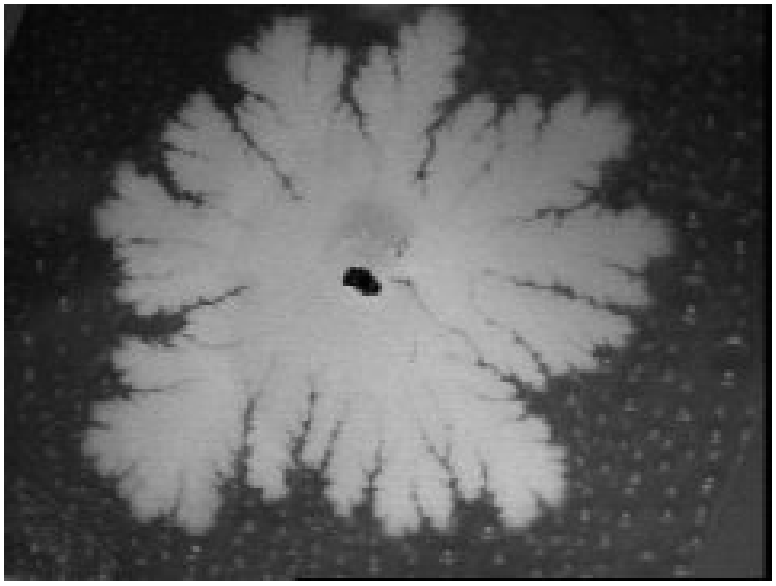

Figure 5.8: Sample E. Coli baterial colonies grown under starvation conditions.

2. Using the **Fractal Dimension** program, find the dimension of the colony. How does the dimension vary as nutrient concentration varies in the plates? Measure both the global dimension of the aggregate (the dimension of the entire colony including the interior), and the surface fractal dimension (the fractal dimension of the edge of the colony).

   How does the global dimension compare with that of a diffusion-limited aggregate (DLA)?

   How does the surface dimension compare with that expected from a random walk? Compare the dimension obtained with that found in HandsOn 17. If the dimension differs from that of a random walker model, does it behave as you would expect with bacteria that are socially cooperative? Write a short paragraph explaining why, or why not.

3. Plot the radius of the aggregate versus time and find the growth velocity. Is this also a function of nutrient level?

4. Is the growth of the colony limited by diffusion of nutrients? Suppose that a nutrient molecule moves an average distance r in a time t according to the *diffusion law*: $r^2 = 4Dt$ where D, called the *diffusion constant*, has the approximate value 10^{-6} cm^2/sec. On average how far can a nutrient molecule diffuse in one hour? One day? How does that compare to the initial and final growth velocities you observed for the bacterial colony?

5. How do you interpret your results for the plate you inoculated at two points 0.5 centimeter apart? Is it consistent with a model in which access to nutrients is controlled by diffusion? What about the growth pattern on the plate inoculated at two points 1 centimeter apart? Can you simulate this experiment using the **Aggregation Kit** program?

END ACTIVITY

HandsOn 21: Staining Bacterial Colonies

If you want to preserve your bacterial colony, you can stain it. Staining the colony kills the bacteria to prevent further growth, and makes the colony easier to look at both with the naked eye and under a microscope.

> **Note of Caution:** Please note that the methanol and acetic acid used in the staining solution are hazardous. To prevent damage to the mucus membrane, a fume hood should be used. Gloves should be worn to prevent skin irritation and neither chemical should be ingested. Be sure to keep the Material Safety Data Sheets provided by the chemical supply company. You will also be working with a strong staining agent. Be sure to take precautions regarding skin and clothing.

You will need:

- stirring rod
- beaker
- graduated cylinder
- 0.1% Coomassie Brilliant Blue R stain (available from Sigma)
- methanol
- acetic acid (or vinegar)
- distilled water
- Staining Solution
- Rinsing Solution

To prepare 1 liter of staining solution (be sure to follow the exact order of these steps):

1. To a 1000 ml beaker add 400 ml distilled water.
2. Add 500 ml methanol.
3. Add 100 ml acetic acid. If acetic acid is not available, vinegar can be used in its place.
4. Add 1 gram of 0.1% Coomassie Brilliant R stain powder. Mix thoroughly until the solution becomes a uniform blue.

The ingredients can be scaled proportionally for smaller amounts of staining solution. Use the base proportions of (4:5:1) distilled water:methanol:acetic acid.

The rinse solution can be made by following STEPS 1-3 for making the staining solution. Be sure not to add the stain powder to your rinse solution.

1. Pour staining solution onto plate. Solution should just cover the surface of the nutrient agar. Let the staining solution stand on the nutrient agar for approximately 45 seconds. This may vary depending on the size and color of the colony that you are staining.
2. Pour solution off the nutrient agar. Pour on rinse solution. Agitate rinse solution for approximately ten seconds. Let the rinse solution stand on the nutrient agar for approximately 50 more seconds.
3. Pour off the rinse solution. If any rinse solution remains, you can invert the plate onto a paper towel until it is dry.

Note of Caution: When disposing of unwanted bacterial colony plates, you must first autoclave them. This will kill the bacteria, and make the plates safe for disposal in a regular trash bag.

END ACTIVITY

5.2 Termite Nesting and Foraging in Two Dimensions

To someone sitting out in the sun watching the ants, bees, and beetles on a hot day, it appears that insects roam about in a random fashion. The trails they leave in the dust are less orderly than what we imagine the interiors of their nests must look like. Not only ants make nests; many other social insects do so as well. In nests of such insects, especially the highly organized insects such as termites, wasps, and bees, decisions appear to be made cooperatively, as if in a highly organized society.

The more primitive termites feed directly on the wood in which they nest, while advanced species of termites nest in soil and forage for dead wood, grass, seed, and other sources of cellulose, a major component of most plant cell walls. To reach this food, workers make tunnels through the soil, construct covered trailways over the surface of the ground, or mark exposed trails with odor for others to follow.

Studying such insects in the wild is a task for dedicated entomologists. However, it is possible to get a flavor for the nesting and foraging behavior of such social insects using simple apparatus. Below we describe such an experiment using termites.

In the experiment that follows, you study termites forming trails. The termites are confined to sand between two plates of acrylic plastic as shown in Figure 5.9. The plates are spaced 1/32 inch (0.08 centimeter) apart, corresponding to the widest part of a termite's body, its head. You place about 150 termites at the center of the cell, and from there they forage in the sand seeking food. There is no food in the sand (although you are invited to alter the experiment to include food—see research activities at the end of this chapter).

A fundamental question we try to answer is whether or not we can distinguish the termites' foraging behavior from random motion, and whether or not there is a quantitative way to evaluate the patterns of their trails.

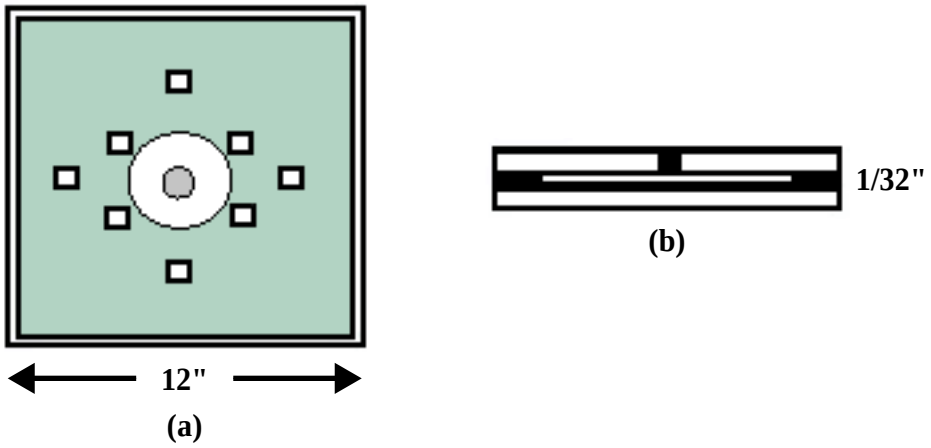

Figure 5.9: The termite cell. (a) Top view of cell. Termites are placed at the center and move into the shaded area that is filled with sand. (b) Side view of termite cell, indicating 1/32 inch (0.08 centimeter) spacing between plates.

Q5.8: Try predicting the pattern of termite tunnels as the termites spread out from the center of the cell. What would this pattern look like if the termites were executing a random walk starting from the center?

Instructions for building termite cells are available on our Web site and on the accompanying CD-ROM.

HandsOn 22: Termite Tunnel Patterns

The process of finding wild termites (as opposed to domesticated termites) varies by geographic region. In New England, we go to a wooded park and search among the remains of dead trees on the ground. Termites like soft, damp wood. An ax, a hammer with a good claw, or a crowbar are useful tools to pry or split open rotting wood. You will encounter many different insects during your search, in particular carpenter ants, but with luck you will find termites. The best time to find termites is during warm weather. In the fall termites go underground to escape the cold winter. (What can be so fine as a day in June hunting termites with your hatchet?) In some cases it is possible to order

termites from biological supply companies. The termites we find locally are the species *flavipes reticulitermes*.

Once a colony is found, collect the pieces of wood and place them in plastic trash bags. If possible, also collect some of the earth surrounding the piece of wood. It is a good idea to use a double bag (one bag inside another) to prevent tears and the subsequent escape of the termites.

When you get the termites back to the laboratory, place the logs in plastic trays, boxes, or tubs lined on the bottom with the dirt you collected. Be sure to drill or puncture holes in the lid of the container for ventilation. Periodically the wood should be sprayed with water to maintain an adequate moisture level. The greatest threat to the colony is dehydration. If you plan on keeping the termites for a period of time, use a mold inhibitor in the spray. This will prevent mold from growing on the wood, which can kill the colony.

To create termite tunnels in the laboratory, follow this procedure:

1. Follow the detailed instructions in order to construct your termite cell. We suggest that a team of "shop-savvy" students perform this task together.

2. Moisten a cotton ball with acetone and lightly wipe the inner surface of the cell. Allow a few minutes for evaporation. This will remove fingerprints and any micro-organisms remaining on cell.

3. Fill a beaker or other container with sand, and slowly sift the sand over the bottom of the cell until the surface is evenly covered. Level the sand with the shim around the outer edges. This can be accomplished by using your top acrylic plate as a straight edge to smooth out the sand. Hold the top sheet perpendicular to the cell and slowly drag it across the surface. You will know when the sand is flat because very little sand will be displaced as the plate is dragged along the cell.

4. Moisten the sand with water using a spray bottle. The sand should be wet, but not so wet that pools of water accumulate on the sand. About 1 fl oz. of water is the right amount.

5. Secure the top of the cell to the bottom using by placing bolts in each of the holes that you drilled. Tighten down the bolts with the wings nuts. Be careful not to disrupt the smoothness of the sand.

6. Place tape along the entire side of the cell where the top and bottom plates meet.

7. In the center of the bottom plate of a small petri dish, drill a hole the diameter of the hole on the top plate of the termite cell. This would be 1/2" if you strictly followed the instructions above. Tape this bottom portion of the petri dish onto the top plate of the cell with its hole coincident with the cell's hole.

8. **Inserting Termites into the Cell:** When handling the termites, do not expose the termites to excessive light, heat, or air for any significant length of time. This is particularly important if you decide to use an overhead projector to display the pattern that forms. This works, but remember that dehydration is a threat to the survival of the termites and overhead projectors produce a large amount of heat.

    a. Break open a piece of wood over a tray or empty container. If there are termites, they will fall harmlessly onto the tray. Using a pair of termite forceps (specially designed forceps that prevent excess pressure at the tips), carefully pick up a termite and place it into a clean small petri dish. You can use the top of the petri dish which was taped to the cell top. Repeat this process until you have roughly 100 to 200 termites. If you have a scale available, about 1 gram of termites is the correct amount.

    b. Occasionally, pieces of wood will end up in the petri dish. Make sure that the pieces are removed before proceeding.

    c. Pour the termites into petri dish taped to cell and cover this petri dish. When most of the termites have entered the cell, you can push the stragglers over the edge and remove the petri dish.

9. Keep the cell in a warm but not hot place, and not in the sun. Termites are nearly blind, so light shouldn't disturb their activity. However, they dehydrate very easily, and they are easily cooked in a strongly illuminated cell.

10. After about a day, most of the termites have descended into the damp sand. Kick the stragglers over the edge, remove the petri dish, and cover with tape.

11. It takes one to three days for the pattern to develop. Take frequent pictures at regular intervals. If possible, use a video camera to grab images. Alternatively, trace the developing pattern as a function of time on a piece of paper, or scan the pattern into the computer. In any case, keep records of the overall radius of the growing pattern as a function of time.

12. Watch the termites carefully. Termite society is divided into different classes or castes. Can you distinguish the different castes and the tasks each caste carries out? How do they communicate? How do they make the tunnels? Do their tunnels intersect with one another? What keeps the sand from collapsing into the tunnels? Do you see any preferred directions to their activity? If so, can you reason why they have selected these directions?

You should remove the termites from the cell if:

- They have reached the edge of the sand and appear to have completed a pattern. (Note: Over longer periods of time the pattern may change further.).
- No significant pattern was created after the first week (varies by colony).
- The sand in the cell appears to be excessively dry.

1. Unscrew and remove the top plate of the cell. Almost all the termites will remain on the sand. You might find that some will stick to the top plate. If this is the case, invert the top plate and set it aside. Your main concern is the large mass of termites still in the sand.

2. The sand should still be sufficiently moist to be able to incline the bottom plate without the sand running off. If you incline the bottom plate vertically, the termites will fall out of the cell. Do this over the wood/dirt where you maintain your colony of termites. The few termites remaining can be removed with forceps.

3. After the termites in the bottom plate are removed, remove the termites on the top plate.

4. Clean the bottom and top plates. Before using the plates again, let them dry for 24 hours. The plastic plates absorb moisture from the sand, and warp slightly when they are separated. The plastic must be allowed to thoroughly dry.

Data Analysis

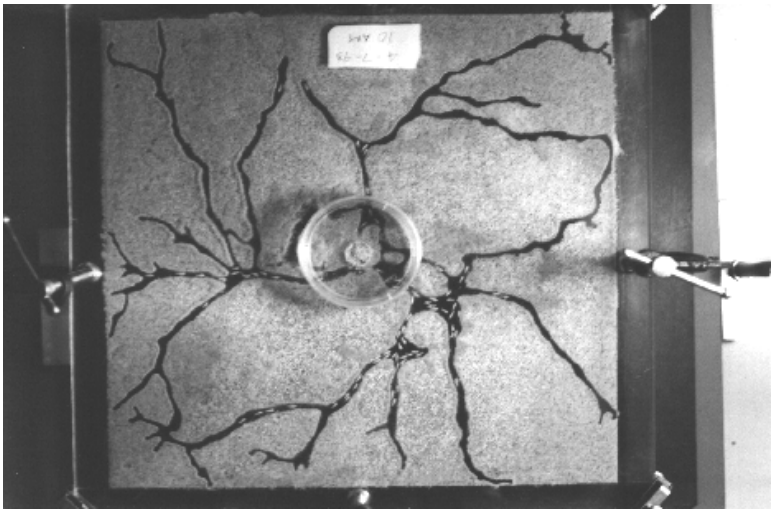

Figure 5.10: A typical termite colony after several days growth.

1. Your analysis should come from your observations as shown in Figure 5.10. Do the termites communicate? Do you think this affects their tunneling? Do you see evidence that later termites follow a path of odors ("pheromones") laid down by earlier termites?

2. Is the pattern random? the product of a diffusion process? Are the termites effectively ballistic—do they move along a single straight line? Is it necessary for them to search every place in the cell?

3. Suppose the termites move like randomly-walking molecules. If the average radius of the foraging pattern grows to ten centimeters in one day, how long would you expect it to take for the pattern to grow to a radius of 20 centimeters? Twice as long? Four times as long? Do your observations match your prediction?

4. Using your scanned or video-captured images, measure the fractal dimension of the pattern at different times. Follow the same steps you used in Chapter 3 by first turning your image into an digital image file and then find the dimension with the **Fractal Dimension** program.

END ACTIVITY

5.3 Fractal Root Systems

Plant roots are classic examples of plant life. Every plant has a submerged branching system designed to support its life functions. But why do roots branch? And why do they branch at so many different levels, that is, why are there branches of branches? Is this the most efficient method for a plant to obtain nutrition?

> Q5.9: Can you think of reasons for the similarity between the branching pattern of root systems and the patterns controlled by diffusion processes?

Root growth occurs by elongation of the root tip. Cells in the growing root divide in a region of tissue known as the meristem at the interior of the root. Growth begins as soon as the seedling germinates. The root that appears from the seedling or embryonic plant is known as the *primary root*. However, if you have ever noticed a plant or seedling after a few weeks, there is more than one vertical root; these other roots

are called *secondary roots*, and branch from the original primary root. But roots do not all grow straight in a vertical direction; many parts of the root extend sidewise from the primary or secondary roots. These roots are known as *lateral roots* and originate from the same tissues as the primary root.

Many factors affect the growth of plant roots. Of particular interest is the resistance due to the physical barrier of the soil. Properties like the compactness of the soil can alter root growth. For example, in compact soils, the spaces between soil particles, are reduced in both number and size. The space size is usually referred to as *pore size*. To force their way through compact soil, roots thicken and their rate of growth—elongation—decreases. Their branching patterns become modified and often there appears to be an increase in lateral root growth. (The lateral roots are often smaller in diameter than the primary root, and are better able to grow through tighter pores.) There is much controversy about the variety of mechanisms plants develop in response to soil resistance.

> Q5.10: Compare root growth and branching to the growth and branching of viscous fingers in the Hele-Shaw experiment of HandsOn 15 and HandsOn 16). Do the reasons for branching of viscous fingers apply also to roots? Does the living root system have a "purpose" that viscous fingers don't have? Does randomness play a part in both?

Many other environmental variables affect root growth. In poorly aerated solutions, roots tend to grow straighter, shorter and to generate more lateral roots. In corn plants, light also alters root growth. Typically, blue light inhibits cell elongation and multiplication, while red light inhibits only cell elongation.

In carrying out the experiments described below, you play detective and try to understand some of the factors that give rise to root structure. You can use what you learned earlier about diffusion and branching in non-living systems. Similar branching structures are observed in snowflakes and during crystallization of minerals. In each

case it is a diffusion process that controls the rate at which growth occurs. The branching pattern apparently results from this diffusion. For growing crystals, minerals diffuse toward the growing surface. For snowflake growth, heat is released as the ice forms and this heat must diffuse away from the growing surface. For root growth, nutrients must diffuse toward the growing root. This diffusion takes place in a solution for hydroponic growth (growth in fertilized liquids), and in the soil.

It is difficult to go out into the field to do experiments on roots, because when plant roots are removed from the soil they are disrupted. One solution to this problem is adopted in the experiment described below: to use a *rhizotron*, a clear-walled chamber through which to observe roots as they grow.

HandsOn 23: Building a Rhizotron and Germinating Seeds

A rhizotron is a clear-walled chamber through which one can observe roots as they grow. Here we describe two alternative methods for studying root growth with simple rhizotrons. Method 1 involves the use of the rhizotron with a stagnant solution such as water or nutrient mixture. Method 2 involves the use of the rhizotron in a tub of solution with a fish tank aerator providing oxygen to the solution. Several variations in the concentration of nutrient solution are possible in performing the root growth experiment.

In addition to the materials needed to build the rhizotron itself, you will need the following materials:

- petri dish
- filter paper or paper towel
- Seeds (peas, squash, sunflowers, etc.)
- Nutrient solution

The seeds must be germinated before they are placed in the rhizotron. Germination allows the seeds to grow a large enough root system so that they can be supported by the top edges of the rhizotron. Otherwise, the seeds are too small to place on the rhizotron.

1. Soak a few sheets of paper towel or filter paper in water.
2. Place some of the wet sheets of paper towel in the bottom of a petri dish, and place the seeds on top of this.
3. Cover the seeds with more wet paper towel, and put the cover of the petri dish on. Store in a warm, dark place. (Placing the petri dish in a black garbage bag to insulate it from heat and keep it out of the light works.)
4. On a daily basis, moisten the seeds and paper with water.
5. The seeds should be allowed to germinate until they are large enough to sit at the top of the rhizotron frame, supported by the plastic sides. The average germination times for commonly used seeds are: Peas—3 days, Barley—4 days, Beans—4 days, and Tomatoes—5 days.

Method 1: Root Growth in Stagnant Solution

1. Mix a 20-20-20 solution of "Peter's Professional Fertilizer." For this concentration use 1 tablespoon fertilizer with 1 gallon of water.
2. Fill the rhizotron with the nutrient solution so that the solution reaches the top edges of the rhizotron.
3. After the seeds have been sufficiently germinated, select the largest seeds. Carefully place the selected seeds on the top of the rhizotron, and tuck the roots between the plastic pieces. More than one germinated seed can be placed in a rhizotron.
4. (Optional) Wrap a strip of black plastic garbage bag around the rhizotron. Make sure that the top edges of the plastic do not extend past the top edge of the rhizotron.

Method 2: Root Growth with Oxygen

For root growth with oxygen, you will be placing the rhizotron in a tub of water so that the solution may be aerated. This requires that you

have sufficient passage of solution from the inside of the rhizotron to the outside.

END ACTIVITY

HandsOn 24: Growing Roots

In this experiment you compare the branching patterns that arise when a plant is grown hydroponically (with fertilizer in the water) to the corresponding patterns when the plant is grown without fertilizer in the water. During the experiment you take photographs of the root system for measurement of the fractal dimension. Finally, once a week for 1–2 months, you record the image of the root patterns for a fractal dimensional analysis of your root system.

When you record your observations at each date, you should include in your table:

- Length and number of vertical root branches (this includes both primary and secondary root growth).
- Length and number of lateral root branches.
- Number and length of root hairs on vertical and lateral root growth. (These are small and difficult to observe, so use a magnifying glass to find the hairs.)

  You grow roots in a *rhizotron*, two parallel sheets of plastic enclosing a narrow sheet of water and nutrients. This forces roots to grow primarily in two dimensions, not three dimensions as they would naturally. Discuss ways that this might affect the results. Do you expect it will increase or decrease the fractal dimension of the resulting root pattern? What experiment could you use to provide analysis of the full 3-dimensional natural root system?

1. Create two groups of rhizotrons and seedlings. Be sure that both groups consist of the same species of seedling.

   a. Group A - using fertilizer and tap water to fill the rhizotron(s).

   b. Group B - same as the control group but no fertilizer.

Use fertilizer with the mixture 20-20-20, which can be found in any local gardening store. Mix 1 teaspoon of dry fertilizer in 1 gallon (approximately four liters) of water and fill the rhizotron.

2. Measure and record the root system before you begin the experiment. Indicate the age of the seedling you are using and measure the primary root, secondary root, and root hairs as described above.

3. Carefully place seedling in the rhizotron. (The seedlings are delicate, so be careful not to disturb the roots too much).

4. Try to find a regular time at least once a week to make observations and recordings with your lab partner.

5. If you have a digital camera, take an image of your root system through the rhizotron cell similar to that shown in Figure 5.11. If you have a scanner, take a picture of the root system, and scan the photograph. This should be done periodically, roughly once a week. Be sure to record root dimensions in your lab notebook and save all your images.

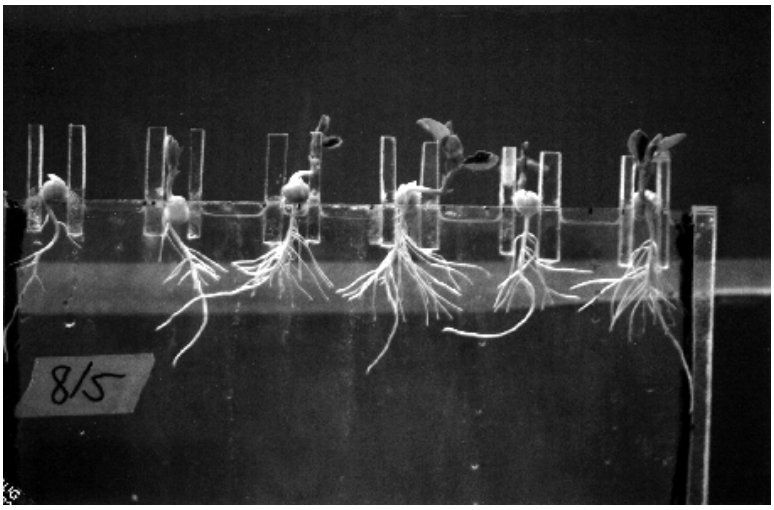

Figure 5.11: Typical root growth results.

6. Keep a record of the color and size of the leaves and stem of your seedling over this period of time. You may also consider checking the pH of your solution periodically. Would you expect this to be different in your groups (A and B) and would you expect this to change over time?

Data Analysis

1. After digitizing your images, use the **Fractal Dimension** program to measure the fractal dimension of your root pattern.

2. From your recorded images, plot the length of the longest root of the pattern *vs.* time and find the root growth velocity. Explain the possible reasons for the shape of your curve. Compare any similarities or differences you observe between groups A and B.

3. From your notebook observations, investigate further the growth velocity of both groups. Plot the length of the vertical roots vs. time and the number of vertical roots vs. time on separate graphs. Do the same plot for the length and number of horizontal roots vs. time.

   After a couple of weeks you may have several root branchings to count. It is easier to plot this information if you average the lengths of the root branchings and average the total numbers of root branchings separately for the vertical and horizontal growths. Plot these averages on your graph.

4. Now find the growth velocities of horizontal (primary and secondary) roots. Explain the similarities and differences you observe or any other observations that you anticipated or that surprised you.

5. What can you say about root patterns and nutrient solutions? What differences did you find in root growth between the two different nutrient solutions?

6. A number of researchers have written computer programs that simulate root growth under conditions similar to those you have

used. How would you use random walkers to create a model of root growth?

END ACTIVITY

5.4 Research Projects

Research projects can be published on our Web site.

Think of your own variants on the experiments of this chapter. Following are a few ideas to get your mind going.

Bacterial Colonies

Vary the agar concentration of the gel. Try 15 g/l (a harder gel), and 7 g/l (a softer gel). What effect would you predict gel concentration will have on the colony pattern? Is your reasoning affected by questions of nutrient diffusion or bacterial mobility?

Make a photo display showing colony shapes as they change with gel and nutrient concentrations.

Termite Colonies

1. Make a mixture of sand with sawdust (say 10% sawdust by volume) and use this to fill the cell (sawdust is food for termites). Do the termites form a different pattern when they have something to eat?

2. Put a piece of damp paper somewhere in the cell. See what happens if the termites locate this food.

3. Put a thin, damp piece of filter or tissue paper on the bottom plate of the cell, and cover it with sand. Does this alter the foraging pattern?

Root Growth

1. Vary the concentration of the nutrient solution by trying serial dilutions, Take your stock solution and dilute it 1/2, 1/4, 1/8, etc.... Based on the research you have done, what effect would

you predict the nutrient concentration would have on the root patterns? Explain your reasoning.

2. Would you expect aerating the rhizotron (bubbling water through it) to alter the pattern you observed in the stagnant rhizotron? Does this have anything to do with nutrient diffusion? If you have the materials, follow the rhizotron set-up discussed on our Web site and on the accompanying CD-ROM, and observe root growth under aerated conditions.

3. Speculate how different wavelengths of light might affect root growth? How could this cause changes in the plant's response to nutrients?

4. Would you expect any differences in root growth if you filled a rhizotron with sand or soil or vermiculite?